# Controlled, targeted, intracellular expression of ribozymes: progress and problems

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Significant progress has been made in the development of ribozymes for a wide variety of intracellular applications, including human gene therapy. Further advances are likely to come from innovative strategies to improve the delivery, expression, co-localization, targeting specificity and substrate turnover of ribozymes. In order to stimulate problem solving in these areas, this article discusses examples of recent successes in intracellular ribozyme applications, and identifies some of the obstacles that remain. In addition, some testable, but as yet untried, ideas for overcoming several of these obstacles are presented.

Just over a decade ago, the study of RNA processing led to the discovery that RNA possesses enzymatic properties[1,2]. The term ribozyme has been introduced to describe RNA molecules with enzymatic activity. A variety of ribozyme catalytic motifs have been identified, all of which catalyze reactions on RNA substrates. These reactions involve site-specific strand scission and ligation reactions. A common feature of all ribozymes is the requirement for a divalent metal ion, such as magnesium, that participates in the chemistry of the reaction. Several different ribozyme catalytic centers have been incorporated into antisense RNAs, imparting the capability to base pair with, and site-specifically cleave, targeted RNA substrates. The enzymatic activity of the ribozyme catalytic center results in the cleavage and destruction of the targeted RNA. Pairing of the ribozyme to the substrate only needs to last long enough for the ribozyme to cleave the targeted RNA, functionally inactivating it (Fig. 1). Once the target has been cleaved, the ribozyme can dissociate from the cleaved products and repeat the cycle of binding, cleavage and dissociation. The ability of ribozymes to cleave targets and then recycle themselves provides an advantage over standard antisense RNAs; these act stoichiometrically, and do not destroy the function of the targeted RNA (Fig. 1). Ribozymes can be produced chemically, biochemically, or biologically, and some of the features of antisense oligodeoxyribonucleotides, such as chemically modified backbones and internucleotide linkages, can be incorporated into synthetic ribozyme molecules, thus imparting added stability to the molecules.

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The specificity of Watson–Crick base pairing, which can be used to direct ribozymes to their targets, suggested from an early stage that these unique molecules could be engineered to recognize and base pair with any cellular or viral RNA target. Within the past five years, there have been numerous reports of site-specific ribozyme-mediated cleavage of a wide variety of RNA targets. The first human clinical trials involving a ribozyme will soon take place for the treatment of HIV infection[3]. In addition to ribozyme-mediated site-specific cleavage, ribozyme-mediated site-specific ligation has been demonstrated[4]. A ribozyme was used to form a functional mRNA from two RNA fragments encoding lacZ *in vivo*.

Although significant progress has been made in ribozyme engineering, there is still a lot that must be learned in order to optimize ribozyme function in the complex intracellular environment. To date, the majority of intracellular ribozyme experiments have been carried out with the hammerhead ribozyme. However, other ribozyme motifs, such as the hairpin, group I intron and the guide RNA for RNAse P, share many of the same basic requirements for maximizing intracellular efficacy. Thus, the successful application of the hammerhead ribozyme should provide valuable lessons for applications of the other ribozyme motifs.

There are five critical areas of investigation that could lead to an increase in the efficacy of intracellular ribozymes. These are: (1) the delivery of ribozymes to the appropriate cells; (2) the efficient expression of ribozymes in these cells; (3) the co-localization of ribozymes in the same intracellular compartment as the targeted substrate RNA; (4) the specificity of the ribozyme to recognize and cleave only its target substrate RNA; and (5) the enhancement of ribozyme-mediated substrate turnover. Strategies for intracellular

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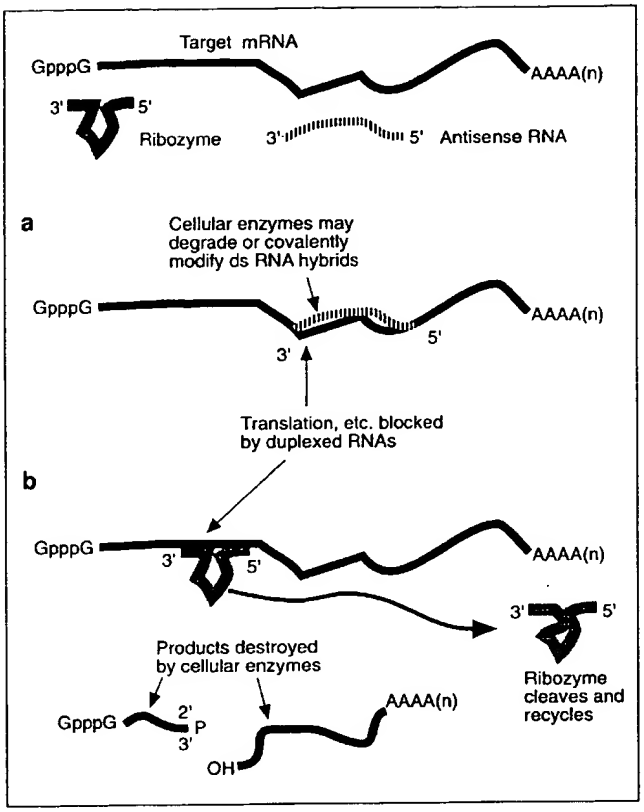


**Figure 1**

Ribozyme versus antisense-RNA-mediated inhibition of mRNA expression. The targeted messenger base pairs with either the antisense RNA (**a**) or the ribozyme (**b**). The antisense inhibitory mechanisms may block cellular functions such as splicing, transport from the nucleus to the cytoplasm, or translation. In addition, the antisense–mRNA hybrid may activate cellular double-stranded ribonucleases or double-strand-specific RNA-modifying enzymes. In either case, the antisense acts stoichiometrically. The ribozyme interacts with the cleavage site by base pairing, cleaves the target and dissociates from the cleaved products; it can then recycle to cleave additional target RNAs. The ribozyme generates a 2′,3′-cyclic phosphate and 5′-OH; this functionally destroys the targeted RNA.

ribozyme applications that address current obstacles in these areas are likely to lead to successful applications for ribozymes. This article focuses on the progress that has been made in intracellular ribozyme applications and the problems that have been encountered. In addition, testable, but as yet untried, ideas for further improving ribozyme efficacy are suggested.

## Ribozyme delivery

The effective use of ribozymes as therapeutic agents depends on devising methods for delivering ribozyme-gene constructs for ribozyme expression to the appropriate target cells (reviewed in Refs 5 and 6). At present, the delivery of gene constructs using retroviral vectors has been the most exploited approach for gene-transfer protocols. These vectors can be engineered to harbor either RNA polymerase II or III transcriptional units for ribozyme expression. However, the general usefulness of retroviral vectors for ribozyme-based gene therapy may be limited by their relatively low efficiencies of transduction into primary cells, random viral DNA integration and the frequent transcriptional silencing of encoded genes.

Other viral, as well as non-viral, vectors developed for gene therapy can be applied to the delivery of ribozymes (Fig. 2). One promising vector that should be evaluated is adeno-associated virus (AAV). This vector has high transduction efficiencies and the potential for integration into a single location on a chromosome[7]. AAV has been successfully used to transduce human cells with an antisense construct that effectively blocked HIV replication in cell culture[8]. Adenovirus should also be considered as a viral vector for ribozyme-gene delivery in situations that do not warrant the integration of the viral vector[5]. Nonviral vectors that can be used for the *ex vivo* delivery of ribozyme-gene constructs and preformed ribozymes include liposomes and cationic lipids[5,6,9]. Ballistic gene guns can be used to propel ribozyme-gene constructs into primary cells that are unreceptive to other methods of transfection[10].

## Ribozyme expression strategies

The choice of promoter sequence context for the intracellular expression of ribozyme transcripts is one of the most important decisions that needs to be made for the successful intracellular application of a ribozyme. The major considerations are the choice of expression system (e.g. inducible, tissue-specific, or constitutive promoters), and the possible adverse or useful effects of *cis*-appended sequences required for RNA capping, transcript termination and export from the nucleus to the cytoplasm. Successful applications of ribozymes that address each of these considerations have been described previously.

Some ribozyme applications may require the temporal control of ribozyme expression. A study of the role of the Fushi tarazu (FTZ) protein at various stages of *Drosophila* larval development used a major heat-shock promoter to provide heat-inducible expression of a ribozyme targeting the FTZ transcript[11]. The selection of this heat-inducible system allowed FTZ to be inactivated at various times during larval development.

The development of a well-characterized bacterial operator–repressor system for use in eukaryotes provides a different type of expression control. Bujard and colleagues have devised a mammalian expression system that uses a combination of prokaryotic and viral regulatory elements to control gene expression tightly[12,13]. The tetracycline (TET) repressor has been fused to the powerful herpes simplex virus (HSV) transactivator VP16. The tetracycline operator elements are positioned upstream of a basal promoter element (Fig. 3). In the absence of tetracycline, TET–VP16 fusion proteins bind to the operator elements and activate transcription from the basal promoter. In the presence of tetracycline, TET–VP16 fusion proteins cannot bind to the operator region, and

transcription is turned off. The tetracycline concentration can be used to modulate transcription levels over several orders of magnitude. This expression system has been successfully used for mRNA expression in a wide variety of cells and organisms as well as in transgenic mice[13–15]. In one transgenic-mouse system, tissue specificity and tetracycline control have been combined by expressing the repressor–VP16 complex from the insulin promoter; this resulted in pancreatic β-cell expression of the transactivator complex[15]. The tetracycline-repressor system may be useful for the controlled expression of ribozymes in a variety of organisms and cell types.

Other ribozyme applications may require the tissue-specific control of ribozyme expression. For example, the development of a transgenic-mouse model for maturity-onset diabetes of the young (type II) required the use of a pancreatic-specific promoter[16]. Selection of the rat insulin promoter ensured that expression of the anti-hexokinase ribozyme occurred primarily in the pancreatic islets.

There are numerous reports in which functionally active ribozymes have been expressed from non-regulated promoters. RNA polymerase II and RNA polymerase III systems have proven efficacious for constitutive ribozyme expression. The cytomegalovirus (CMV) immediate-early-gene enhancer/promoter has been used to constitutively express hammerhead ribozymes that target murine β-2-microglobulin mRNA in transgenic mice[17]. Although the transgenic animals expressed the ribozyme in several different tissues, the most pronounced reduction of the target protein (90%) occurred in the lungs. Therefore, a constitutive promoter does not necessarily guarantee the same ribozyme activity in all tissues.

The nature of the viral vector backbone has also been shown to affect the efficiency of ribozyme expression from its promoter. A comparison of the expression of anti-HIV-1 ribozymes from several different promoters in a retroviral vector has revealed significant differences in ribozyme expression[18]. Optimum functional expression occurred when the ribozymes were expressed as part of a long viral transcript between long terminal repeats (LTRs), rather than as transcripts produced from internal Pol II and Pol III promoters. A possible explanation for these results is that competition between the LTR and inserted promoters occurs in some retroviral vectors. This could either be due to run-on transcription, or to the relatively short spacing between the promoter elements.

If transcription from the upstream promoter negatively affects transcription from the downstream promoter, when they possess the same transcriptional polarity, a possible solution may be to design constructs with opposing transcriptional polarity. A $tRNA^{Val}$ gene has been successfully used to drive the transcription of an anti-HIV-1 hairpin ribozyme in a retroviral vector when the ribozyme was transcribed in the opposite orientation to transcription of the retroviral LTR (Refs 19,20). Promoter competition could still be a problem for transcripts of opposing polarity in retroviral constructs. Nevertheless, this finding suggests that a well-defined transcription unit (e.g. Pol III) in the opposite orientation to LTR transcription may be an important strategy for driving ribozyme expression from retroviral vectors.

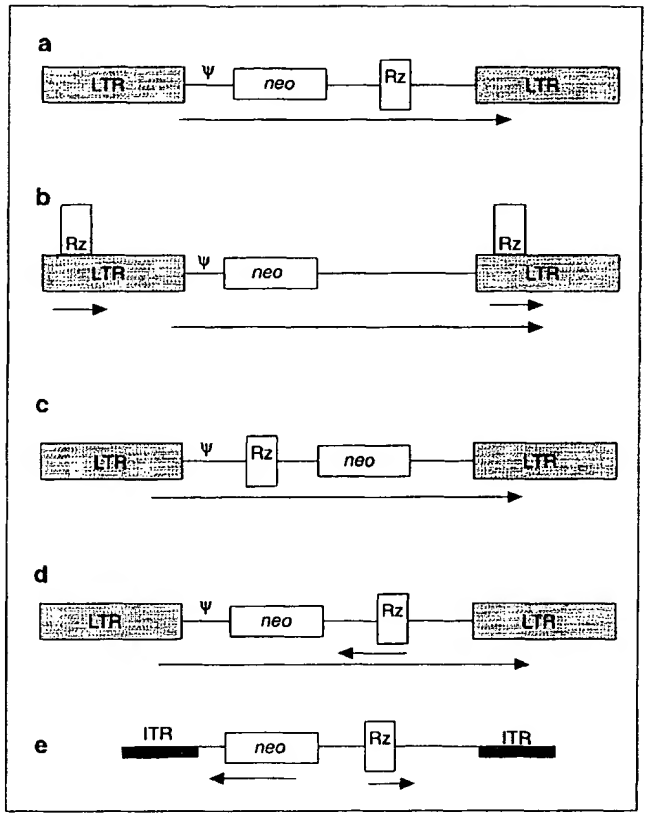


**Figure 2**

Some viral vector constructs for ribozyme delivery and expression. **(a)–(d)** depict different versions of retroviral vectors. In **(a)** and **(c)** the ribozyme (Rz) is expressed as part of the retroviral LTR (long terminal repeats) to LTR transcript. In **(b)**, the ribozyme is driven by a polymerase III (Pol III) promoter that is inserted as a double copy in the LTRs of the viral vector. In this construct, the direction of transcription is the same as that of the viral LTR promoter. In **(d)**, the Pol III–ribozyme construct is transcribed in the opposite direction to the viral LTR, as described in the text. In **(e)**, expression of a ribozyme from an adeno-associated viral vector (AVV) is illustrated. The inverted terminal repeats (ITRs) have weak promoter function, but are not used to drive the ribozyme transcript. The ribozyme can be readily transcribed in either orientation using Pol II or Pol III transcriptional units. The arrows represent the direction and extent of transcription. Neomycin phospho-transferase (Neo) inactivates neomycin, making cells resistant to this drug. Ψ denotes the retroviral packaging signal.

Two tools that have recently been described for the expression of short RNAs are the mammalian U6 small nuclear RNA (snRNA) and adenoviral-VA 1 promoters[21,22]. These are both RNA polymerase III transcriptional systems in which the promoter elements are located upstream of the mature coding sequences. An important feature of both these promoter systems is that a minimal amount of *cis*-appended sequence is required to produce properly

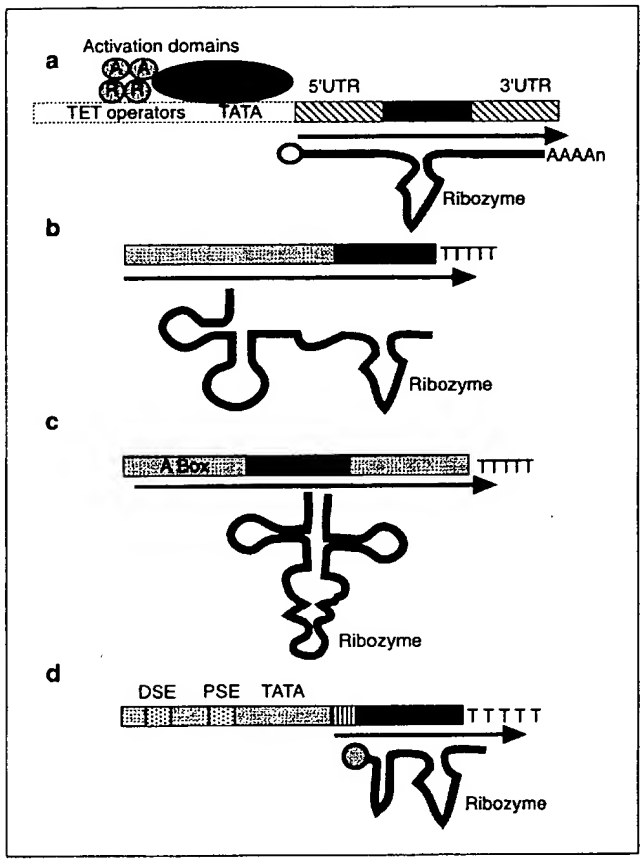


**Figure 3**

Promoter systems for the intracellular expression of ribozymes. (**a**) depicts fusions of the tetracycline (TET) repressor (R) with the herpes simplex virus (HSV) transactivator VP16; fusions are bound to the TET operator that is positioned upstream of a basal promoter. In the absence of TET, the complex binds the operator region, activating transcription. In the presence of TET, transcription is turned off because the repressor interacts with the antibiotic and can no longer bind the operators. (**b**) and (**c**) represent Pol III promoter cassettes derived from a tRNA gene. The ribozyme either replaces part of the pseudouracil stem-loop and aminoacyl-acceptor stem (**b**), or is inserted into the anticodon loop (**c**). In both cases, intact A and B boxes are required for expression; a string of 5 Ts (Us in RNA) terminates transcription. (**d**) shows a representation of the mammalian U6 small nuclear RNA (snRNA) gene promoter (adapted from Ref. 21). This is a Pol III promoter that is similar to the tRNA gene; however, the promoter regulatory elements are upstream of the mature coding region. The ribozyme construct is positioned immediately after the capping signal (a small stem-loop structure and a short stretch of adjacent nucleotides) so proper capping of the transcript can take place. Transcription termination is signalled by a region of 5 Ts (Us in RNA). DSE represents the distal-sequence enhancer, and PSE the proximal-sequence element. The Pol II transcripts and the U6 transcripts are capped, whereas the tRNA transcripts are not. UTR denotes untranslated regions.

initiated and terminated transcripts. In the case of the U6 system, approximately the first 30 nucleotides of the mature U6 coding sequence are required to cap the transcripts with γ-methyl phosphate[21]. As with all Pol III transcriptional units, the termination signal is a stretch of five uridines that follows the inserted coding sequence. The expression of ribozymes by the human U6 promoter, in which the capping signal of the U6 sequence was appended to the ribozyme transcript, resulted in the functional inhibition of HIV-1 replication in a co-transfection assay (D. Engelke, J. Zaia and J. Rossi, unpublished). Although it has not yet been rigorously demonstrated, the RNA cap provided by U6 may impart both stability and nuclear localization to heterologous RNAs that are inserted downstream.

### Co-localization of ribozyme and target RNAs

Strategies for enhancing the accessibility of the target RNA to pairing with the ribozyme in a complex intracellular environment merit more intensive investigation by those interested in optimizing the intracellular ribozyme function. The best illustration of this point comes from Sullenger and Cech, who demonstrated that co-localization of a ribozyme with the target RNA greatly improved the efficacy of a hammerhead ribozyme[23]. The ribozyme and target were co-localized via the retroviral dimerization domains, forcing encapsulation (co-packaging) of the retroviral transcript encoding the ribozyme with the retroviral RNA encoding the target *lacZ* gene; the ribozyme was only effective when the RNAs were co-packaged. There are many examples of ribozymes functioning in an intracellular environment, but this was the first deliberate attempt to study the relationship between ribozyme efficacy and co-localization of a ribozyme and its target. Although this represents an extreme example, a good case can be made for developing strategies to co-localize the ribozyme transcript to the same intracellular compartment(s) as the targeted RNA.

Very little is known about the mechanisms that regulate the pathway of movement from transcription to translation for most RNAs. There is increasing evidence, albeit controversial, that nuclear transcripts are processed and migrate along specific tracks; this predicts non-uniform distributions of specific nuclear transcripts (reviewed in Ref. 24). In addition, there are numerous examples of messenger RNAs that localize to specific regions of the cytoplasm. The best studied examples of localized RNAs are the oocyte and early embryo mRNAs of *Drosophila* and *Xenopus* (Ref. 25). Other mRNAs, such as actin mRNA, have been shown to localize to cytoskeletal components[26,27].

The 3′ untranslated region (UTR) contains the signal that is responsible for the localization of many of the mRNAs that have been studied[25,27]. Messenger RNAs encoding two actin isoforms (β-cytoplasmic and α-cardiac) were shown to occupy different compartments within the same cytoplasm. Moreover, the sequences in the respective actin 3′ UTRs were sufficient to localize an mRNA encoded by *lacZ* to the same cytoplasmic compartments[27]. It should be possible to test whether such intracellular localization signals will enhance ribozyme targeting by directing ribozymes to the same intracellular compartments as the target mRNAs. The same 3′ UTR sequence that is present in the target mRNA can be appended to a

ribozyme transcript. If this strategy improves the co-localization of ribozyme and RNA target, it may enhance ribozyme function.

Co-localization strategies could take advantage of various post-transcriptional processing events that take place in the nucleus of the cell. The insertion of *trans*-acting ribozymes into RNAs that are directed into the nucleus (e.g. the small nuclear RNAs involved in splicing) can be used to concentrate ribozymes within the nucleus. Co-localizing ribozymes with mRNA in the nucleus may help to increase the probability of ribozyme–target interaction before translation occurs in the cytoplasm.

For many targets, the best compartment for interaction with a ribozyme can only be determined empirically. Therefore, it is important that several different strategies are tried in order to determine which is most efficacious for a given ribozyme and target combination.

## Intracellular targeting and substrate turnover by ribozymes

Base-pairing specificity determines the selectivity of a ribozyme for its target RNA (substrate). As every ribozyme sequence has different potential base-pairing interactions, an accumulation of data from many different ribozyme experiments will be required for a rigorous assessment of the optimal combinations of sequence composition and base-pairing arm lengths. Base-pairing mismatches or mutations at, or adjacent to, the site of cleavage can also affect ribozyme efficacy. This problem is especially significant when designing ribozymes against genetically variable targets such as HIV. One strategy already being tested for ribozyme-mediated inhibition of HIV is the simultaneous use of multiple ribozymes to two or more targets[18,28]. This may help to minimize any loss of ribozyme activity due to base-pairing mismatches and mutation of the cleavage site.

There is no optimum region for cleavage that is generic to all RNAs. The GUC triplet is frequently chosen for *trans*-acting hammerhead and hairpin ribozymes because of its wide occurrence in natural ribozyme motifs. Other triplets are also potential targets, but the efficiency of cleavage seems to vary depending on the context of the sequence containing the triplet[29,30]. The use of RNA-folding programs to analyze intramolecular structure can be useful for choosing a cleavage site. An even more powerful approach is to scan the accessibility of several potential intracellular target sites along the length of the message. This approach has proved useful for synthetic oligodeoxyribonucleotides, but has not yet been systematically carried out with synthetic ribozymes.

All RNAs interact with cellular proteins from the moment they are synthesized until they are degraded. Some of the cellular RNA-binding proteins are more abundant than the histone proteins (reviewed in Ref. 31), and their interactions with ribozymes and substrates will influence the intracellular functioning of ribozymes. The heterogeneous nuclear–ribonuclear protein hnRNP A1 is associated with RNAs in the nucleus and cytoplasm. When purified and incubated with ribozyme and substrates *in vitro*, hnRNP A1 readily associates with these RNAs, facilitating their interaction and the release of cleaved products from the ribozyme base-pairing arms[32,33]. These proteins facilitate ribozyme turnover by enhancing product release. It is highly likely that intracellular proteins such as hnRNP A1, and other similar RNA-binding proteins, are good candidates for enhancing intracellular ribozyme activity. Enhancement may be restricted by the stability of the base-pairing interactions between ribozyme and substrate as was observed for hnRNP A1 (Ref. 32) and the HIV-encoded nucleocapsid protein NCp7 (Refs 32,34). As with many other aspects of ribozyme function, empirical testing of different ribozyme–substrate combinations is still required so that we can capitalize on the facilitation that is mediated by proteins such as hnRNP A1.

## Future prospects

The hypothesis that ribozymes could be used as agents for inactivating or altering the flow of genetic information was only proposed several years ago, but is now an established fact. However, there is still a lot to be learned about the movement of RNA inside cells and the cellular factors that can impede or enhance ribozyme action so that we can capitalize fully on the targeted RNA-inactivation property of ribozymes. The best approach for optimizing ribozyme function in a complex intracellular environment is to understand as much as possible about the intracellular fate of the RNA that is being targeted. As new techniques in cell biology become available, such understanding will be less problematic.

Basic studies of ribozyme structure and mechanisms of catalysis are flourishing at the academic and commercial level, and it can be expected that many new developments will continue to take place in these areas. Nevertheless, the next frontier for ribozyme applications is that of enhancing our understanding of the cell biology, trafficking and intracellular localization properties of RNA. These are the areas within which we can expect to see most research efforts in the immediate future.

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# Intracellular antibodies: development and therapeutic potential

Jennifer H. Richardson and Wayne A. Marasco

Single-chain antibodies, synthesized by the cell and targeted to a particular cellular compartment, can be used to interfere in a highly specific manner with cell growth and metabolism. Recent applications of this technology include the phenotypic knockout of growth-factor receptors, the functional inactivation of $p21^{ras}$ and the inhibition of HIV-1 replication. Intracellular antibodies are likely to have a widespread impact in biological research as a simple and effective alternative to other forms of gene inactivation; they demonstrate clear potential as reagents for cancer therapy and for the control of infectious diseases.

The specific and high-affinity-binding properties of immunoglobulin molecules have long been used in biomedical science as *in vitro* tools for the identification, purification, or functional manipulation of target antigens. A recent series of papers has demonstrated the potential for using antibodies to interfere with biological processes inside the cell in a highly specific manner. It is well established that preformed antibodies can transiently inactivate a target protein when they are introduced into the cell by microinjection[1]. The concept of stably transfecting cells with the antibody gene, coupled with advances in antibody engineering, have allowed the power of intracellular antibodies to be fully realized. This review summarizes recent experiments in which single-chain antibodies that have been synthesized by the cell and targeted to the relevant cellular compartment, have been used to downregulate growth-factor receptors, to inactivate

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